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Up-Regulation of Hormone Response of Human Papillomavirus Type 16 Expression and Increased DNA–Protein Binding by Consensus Mutations of Viral Glucocorticoid Response Elements

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Human papillomaviruses (HPVs) and steroid hormones are linked to the development of cervical cancer. Studies from our laboratory and others showed that the steroid glucocorticoid and progesterone hormones activated the expression of HPV type 16. This activation was attributed to the specific interaction of the glucocorticoid receptor (GR) with the three glucocorticoid response elements (GREs) in the HPV16 regulatory region. In the present study, we first examined the glucocorticoid response mediated through the GREs, using GRE consensus (GREc) mutations and expression assays from a heterologous basal promoter. Both single and triple HPV16 GREc constructs increased expression in the presence of the dexamethasone glucocorticoid in HeLa cervical carcinoma cells and primary baby rat kidney epithelial cells, in comparison with the triple wild-type GREs. Further, the hormone increased significantly the expression of the viral E6–E7 oncogene mRNA from intact HPV in primary human ectocervical cells in in situ hybridization assays. Three in vitro assays of DNA–protein interaction with oligonucleotides and HeLa cell extracts showed a higher binding of protein to two of the HPV16 GREcs than to the wild-type GREs. This applied especially to the GRE containing an overlapping NF1 half site, that also had a greater differential induction by dexamethasone of expression in vivo. The NF1 site was mutated in the GREc that also was bound by unique, lower-mobility complexes in electrophoretic mobility shift assays. UV-crosslinking assays confirmed the increased binding and showed binding by a 96-kDa protein, probably the GR. Our results show an important role of glucocorticoids in HPV16 expression. The direct action through the HPV16 GREs is suggested to be mediated by the hormone-activated GR in association with other factors.

KEY WORDS: glucocorticoid hormone, consensus GREs, HPV16 transcription, protein–DNA binding

INTRODUCTION

Squamous cell carcinoma of the uterine cervix is the second most common cancer of the female population in the world [Parkin et al., 1993; Brinton et al., 1993]. The association of human papillomaviruses (HPVs) with cervical cancer is supported by numerous studies that indicate the role of these viruses, especially the high-risk HPV types 16 and 18, in the initiation of and progression to cancer [reviewed in zur Hausen and de Villiers, 1994; McDougall, 1994]. In addition to the etiology of HPVs, steroid hormones have been suggested as an important cofactor in malignant conversion [Stern et al., 1977; Bornstein et al., 1995]. The use of oral contraceptives and pregnancies were associated with an increased risk for the development of cervical cancer [Negrini et al., 1990; Brinton et al., 1993; Bohkman and Urmancheyeva, 1989].

Progesterone and glucocorticoid hormones have been shown to have important roles in the regulation of expression of high-risk HPVs, especially HPV16 [Chan et al., 1989; Chong et al., 1990]. The HPV16 cell-specific constitutive enhancer contains binding sites for the glucocorticoid receptor (GR) known as glucocorticoid/progesterone response elements (GREs) [Chan et al., 1989]. In these studies, the GRE sequence at nt 7640 of the HPV16 long control region (LCR) was shown to bind

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TABLE I. Wild-type (WT) and Consensus Glucocorticoid Response Element (GREc) Sequences in Various Constructs*

Plasmid/oligonucleotide	Glucocorticoid response element sequences		
	7385 (83%)	7474 (75%)	7640 (75%)
pHPV(WT)	*attt*GCTACATCCTGTTTT*tgt*	*attt*GGCACAAAATGTGTT*ttt*	TGTACATTGTGTCAT
p3×GRE(WT)	----------	----------	----------
pHPV3×GREc	-----g---------c----	------t---------tc----	g---------tc-
p1×GREc			g---------tc-
p1/3×GREc	----------	----------	g---------tc-
p3×GREc	-----g---------c----	------t---------tc----	g---------tc-
pGRE7385/7385 GRE	----------		
pGREc7385/7385 GREc	-----g---------c----		
pGRE7474/7474 GRE		----------	
pGREc7474/7474 GREc		------t---------tc----	

*The palindrome sequence of GREs of wild type is shown in capital letters. The consensus mutations created by site-directed mutagenesis are in lower case letters, and the other wild-type nucleotides are indicated by dashes. The flanking sequences of the oligonucleotides used for nt 7385 and nt 7474 GREs are in lower-case italics. Wild-type [pHPV(WT)] and triple-consensus (pHPV3×GREc) GREs are in whole HPV16 plasmids; CAT reporter plasmids contain single-consensus GRE (p1×GREc), one-consensus GRE and two WT GREs (p1/3×GREc), triple WT GREs [p3×GRE(WT)], and triple-consensus GREs (p3×GREc). The first nucleotides of each GRE are indicated, and the percentage homology of each GRE to the consensus sequence is indicated in parentheses. The overlapping *tt*GGC (NF1) and TGTGTCA (AP-1) motifs which overlap the nt 7474 and nt 7640 GREs, respectively, are underlined.

GR and the progesterone receptor (PR). Transient expression assays of this GRE in the same report showed activated transcription, using whole LCR and a GRE oligonucleotide placed upstream of a heterologous promoter [Gloss et al., 1987]. Further, the HPV16 GRE mediated an increase in E6–E7 oncogene transcripts in S1 nuclease assays for SiHa cervical carcinoma cells [Chan et al., 1989]. Loss-of-function mutations of the nt 7640 HPV16 GRE led to the identification of two additional GREs in the LCR [Mittal et al., 1993a]. Transient expression assays in HeLa cervical cells suggested the regulatory role of hormones through individual GREs or combinations of the three GREs [Mittal et al., 1993b]. In addition, characterization of these three GREs by in vitro binding assays and by transformation assays implicated a significant, direct role of glucocorticoid hormones in HPV-mediated oncogenesis [Gloss et al., 1989; Mittal et al., 1993b].

The regulation of expression of HPV16 by glucocorticoids is a complex process, as observed generally for transcriptional gene regulation [Mitchell and Tjian, 1989]. Possible additional multifactorial mechanisms involving the composite GRE at nt 7640 and the two other recently identified GREs remain to be revealed. In this study, we examined such possibilities for the role of the three GREs in expression through heterologous and homologous promoters, using GRE consensus (GREc) mutations. Further, the results obtained were supported by three in vitro DNA–protein interaction assays.

MATERIALS AND METHODS

Plasmids, Site-Directed Mutagenesis, and DNA Sequencing

Oligonucleotides containing the GREc mutations at nt 7385 and nt 7474 (General Synthesis and Diagnostics, Toronto) were used in site-directed mutagenesis, as described previously [Mittal et al., 1993b]. The mutations were confirmed by sequencing. The HPV16 nt 6150–7761 fragments and oligonucleotides containing the GREc mutations were inserted at the BamHI site of the pBLCAT2 chloramphenicol acetyl transferase (CAT) expression vector. The construction of the plasmids containing nt 7385 (pGREc7385) and nt 7474 (pGREc7474) GREc oligonucleotides in pBLCAT2 expression vector was as described for the GRE wild-type oligonucleotides (pGRE7385 and pGRE7474). The latter were previously described as pBLGRE5WT and pBLGRE6WT, respectively, and constructed [Mittal et al., 1993b]. The p1XGREc expression vector contains the HPV16 nt 7640 GREc in pBLCAT2 and was constructed and described as pcD4 previously [Mittal et al., 1994]. To insert the GREc mutations into the whole HPV16 plasmid, the nt 6150–863 sequences containing the mutated GREs were ligated to replace those of the wild-type HPV16 plasmid. The names of the constructs, oligonucleotides used, and GRE sequences in each construct are shown in Table I.

Cell Culture, DNA Transfection, CAT Expression Assays, and In Situ Hybridization Assays

HeLa cervical carcinoma cells and baby rat kidney (BRK) cells were propagated in Dulbecco's modified Eagle's medium. Primary cultures of human ectocervical cells (HEC) were propagated, as described previously [Tsutsumi et al., 1992]. Cells were cotransfected with 10 μg of the indicated CAT constructs and 2 μg pHGO GR expression plasmid, as described [Mittal et al., 1993b]. HEC were transfected by lipofection, as described [Felgner et al., 1987]. CAT assays with the indicated constructs in HeLa and BRK cells were done as described previously [Gorman et al., 1982]. Values were normalized for efficiencies of transfection and expression with assays for the cotransfected β-galactosidase expression plasmid. In situ hybridization assays for HEC transfected with whole genomic HPV16 plasmids were used to detect E6–E7 viral oncogene RNA, according to

Lawrence and Singer [1986]. The probe was the HPV16 HpaII-NcoI (nt 507–863) fragment nick-translated with biotin 7-dATP.

Whole Cell Extract Preparation and In Vitro Protein–DNA Binding Assays

Whole cell extract preparation from HeLa cells was as described [Tasset et al., 1990]. Electrophoretic mobility shift assays (EMSAs) used a kit, according to manufacturer's recommendations (Pharmacia). The double-stranded 22-mer GRE oligonucleotide probes indicated in Table I were end-labeled using [^{32}P]dCTP and reverse transcriptase. Southwestern blot assays used the double-stranded GRE oligonucleotide probes, as described previously [Silva et al., 1987], with minor modification. For ultraviolet (UV) crosslinking assays, the GRE double-stranded oligonucleotides nick-translated in the presence of [^{32}P]dCTP and 0.15 mM 5-bromodeoxyuridine were used as probes, and the assays were as described previously [Ausbel et al., 1994].

RESULTS

Steroid Hormone Response of Triple GREcs

A nt 7640 and two additional nt 7385 and 7474 GREs in the HPV16 regulatory region were identified earlier [Chan et al., 1989; Mittal et al., 1993b]. Here, the role of these GREs was studied, following site-directed mutagenesis of one or three GREs in the HPV16 enhancer region to produce GRE consensus mutations, GREcs (Table I). To study the effect on glucocorticoid-mediated expression, the HPV16 regulatory region with the GREc mutations was cloned into the heterologous promoter of the enhancerless pBLCAT2 expression vector. HeLa cervical carcinoma cells and BRK epithelial cells were used, since the expression of HPVs is epitheliotropic [Chan et al., 1989]. A 2.9-fold induction by dexamethasone glucocorticoid hormone of expression from the reporter plasmid containing the three wild-type GREs in p3XGRE(WT) was observed (Table I; Fig. 1A). On the other hand, the p1XGREc construct, containing the single nt 7640 GREc and a mutated overlapping AP-1 site, showed a remarkable 9.4-fold hormone induction of CAT expression. A slightly higher level of induction of 11.0-fold was observed with p1/3XGREc, containing the single nt 7640 GREc and the two other wild-type GREs at nt 7385 and nt 7474. Interestingly, the uninduced expression from both GREc constructs was significantly lower than that of the wild-type GRE construct (Fig. 1A).

To study hormone-mediated expression further, the two additional GREs at nt 7385 and nt 7474 were also mutated to consensus sequences, resulting in triple GREc sequences in the whole HPV16 enhancer (Table I). In HeLa cervical carcinoma cells, these triple GREc sequences expressed from the receptor vector gave a 22.5-fold induction of activity by hormone (Fig. 1A). This induction was 7.8-fold greater than for the wild-type triple GREs-containing p3XGRE(WT). A similar pattern of expression from the different GREc constructs was

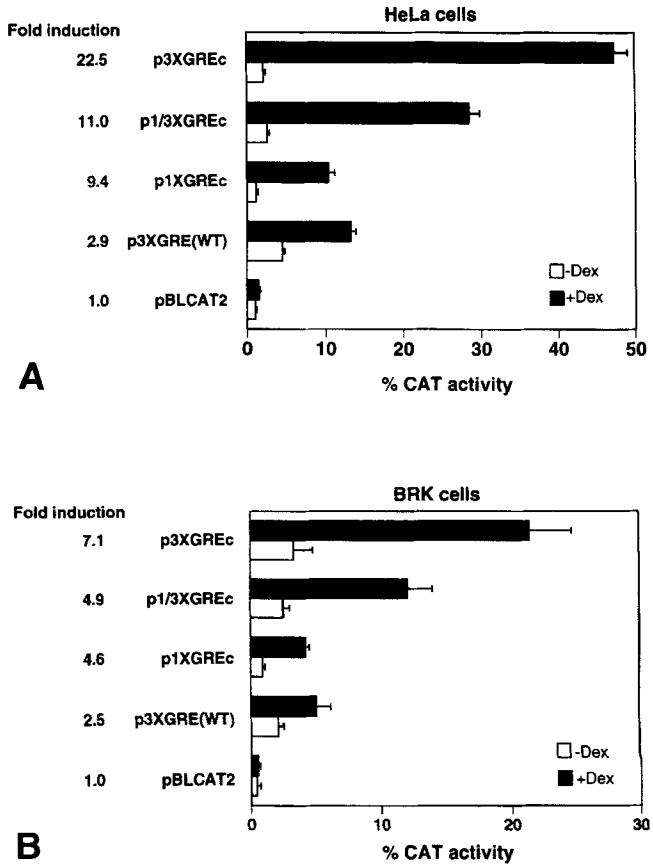

Fig. 1. Effect of HPV16 GREc mutations on hormone response. CAT reporter expression assays were for the GRE constructs indicated in Table I. Assays were in HeLa cells **(A)** and BRK cells **(B)**. The fold induction by 10^{-6} M dexamethasone (+Dex) glucocorticoid hormone was calculated by normalizing the activity with the pBLCAT2 control. The error bars represent the standard deviation of the results of three independent experiments.

observed in primary baby rat kidney (BRK) epithelial cells (Fig. 1B). Although the induction was not as high as in HeLa cells, the GREc constructs gave higher levels of induction than those of the wild-type constructs in the presence of the dexamethasone glucocorticoid steroid hormone. Earlier studies with oligonucleotides of single GREs in expression vectors indicated a role of these sequences in induction by dexamethasone [Gloss et al., 1989; Mittal et al., 1993b]. Here, the in vivo activity of the nt 7385 and 7474 GREs and GREcs was assayed after cloning the oligonucleotides of these sequences (Table I), into the pBLCAT2 expression vector. As shown in Table II, the nt 7385 GREc construct exhibited about 1.5-fold higher induction than the 7385 GRE construct. Moreover, the induction level was 2.3-fold higher with the nt 7474 GREc than with the 7474 GRE construct.

Steroid Hormone Response Via GREcs of Intact HPV16 DNA in Cervical HEC

To examine the effect of the HPV16 GREcs on the expression of the viral E6–E7 oncogenes and the effect in the context of the whole genome, the enhancer region

TABLE II. Effect of HPV16 GREc Mutations on Hormone Response for nt 7385 and nt 7474 GRE Oligonucleotides*

Plasmid	GRE	Induction of CAT activity
pBLCAT2	Nil	1.0
pGRE7385	nt 7385 GRE	2.5 +/− 0.3
pGREc7385	nt 7385 GREc	3.7 +/− 0.2
pGRE7474	nt 7474 GRE	2.1 +/− 0.2
pGREc7474	nt 7474 GREc	4.8 +/− 0.3

*The assays for CAT activities for induction by dexamethasone were as described in the text and the legend for Figure 1A,B. HeLa cells were used for the three independent transfections. The sequences of the GRE and GREc oligonucleotides are indicated in Table I.

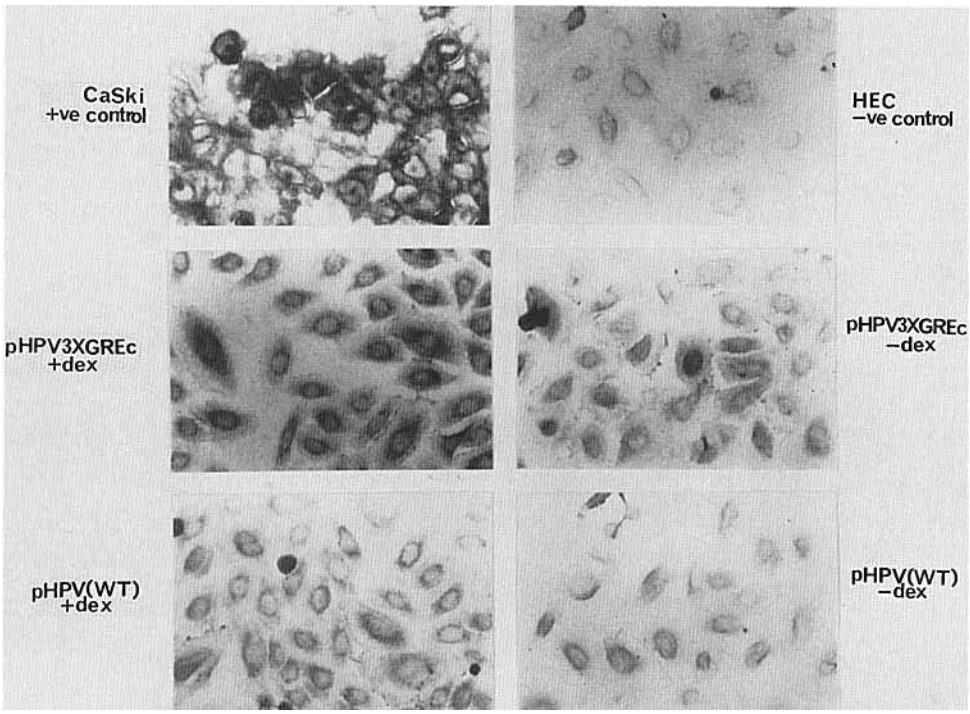

Fig. 2. Effect of hormone on E6–E7 expression in HEC. HEC were transfected with HPV16 plasmids containing wild-type (WT) GREs or triple GREcs (pHPV3×GREc), fixed after 48 hours, and probed with biotinylated HPV16 E6–E7 DNA. CaSki cells containing and expressing HPV16 DNA were used as a positive control, and untransfected HEC were used as a negative control. The transfected HEC were incubated with hydrocortisone-free medium with 10^{-6} M dexamethasone (+dex) or without dexamethasone (−dex). Magnification: ×100.

containing the triple GREc mutations was inserted into the whole HPV16 wild-type plasmid and called pHPV3XGREc (Table I). The constructs were transfected into primary HEC cultures derived from the ectocervix. In situ hybridization for the viral E6–E7 oncogene RNA was used to assay the expression from the homologous HPV16 early promoter/enhancer. The transfected DNA remains episomal during the assays, mimicking the HPV16-infected premalignant cervical cell lesions found in women. For the wild-type (WT) GREs or triple GREcs, expression was significantly higher for the dexamethasone hormone-induced cells (Fig. 2, + dex). An analogous observation was made previously, when HEC were transfected with a single GREc-containing HPV16 plasmid and probed with whole HPV16 for the viral RNA message [Mittal et al., 1993a]. Further, the cells transfected in this study with HPV16 carrying the three GREc mutations displayed stronger signals of hormone-induced E6–E7 message, in comparison with the cells transfected with the wild-type HPV16 plasmid (Fig. 2). Although the assays are qualitative, the response to hormone was signficantly higher than that for the wild-type.

In Vitro DNA–Protein Interaction of Two HPV 16 GREcs

Glucocorticoid hormones initiate the transcription of target genes by diffusing into the cells and interacting with intracellular GR, which then binds the GREs in the enhancers [Pater et al., 1994]. To test the presence, the amount and the nature of factor(s) binding to the GREcs of HPV16, DNA–protein interaction assays were performed. First, EMSA mobility shift assays were performed to test the total proteins that bind, using oligonu-

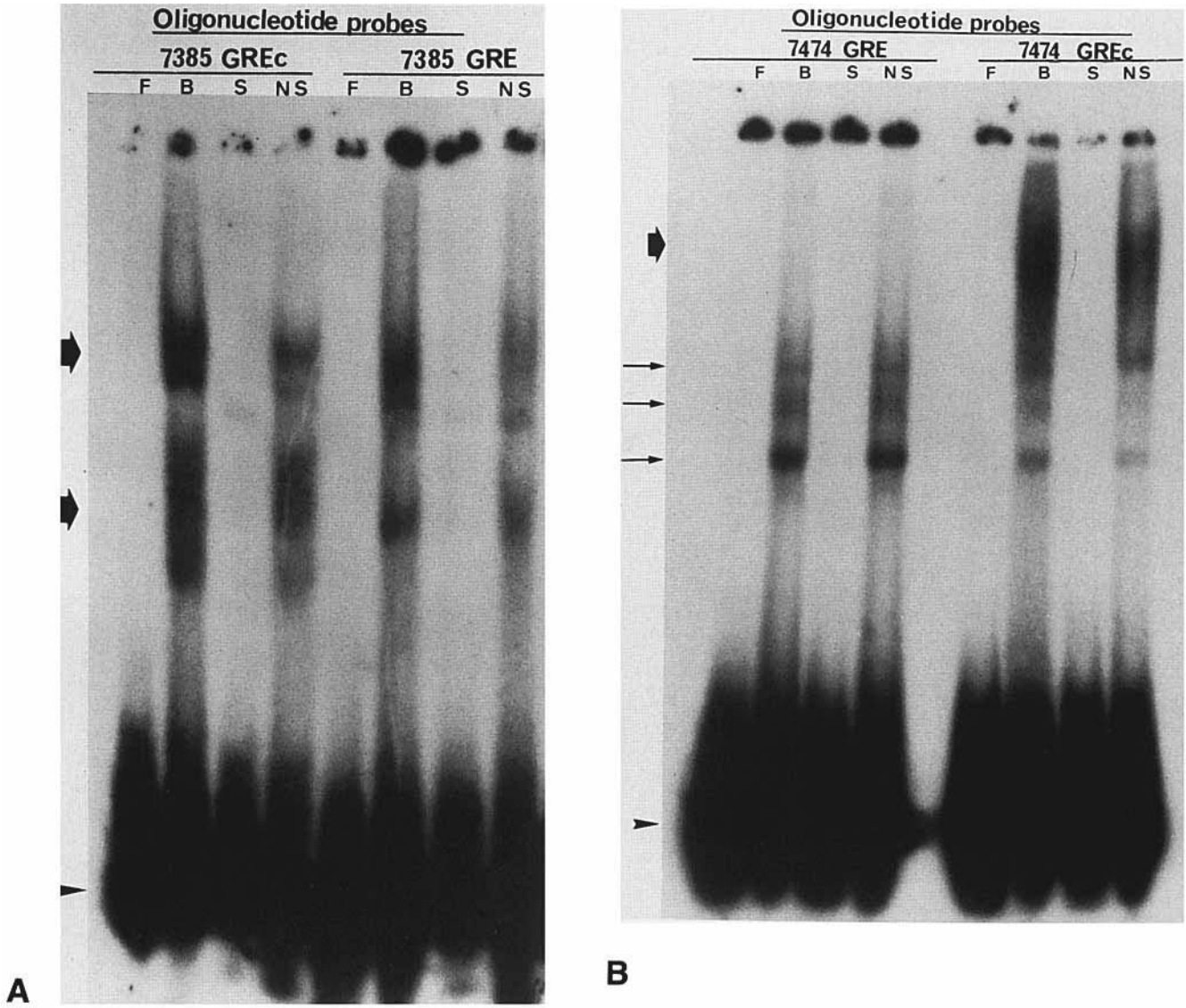

Fig. 3. In vitro protein–DNA interaction of wild-type GREs and GREcs. EMSAs for 10 μg protein in HeLa whole cell extracts were incubated with 20,000 cpm of end-labelled probes. EMSAs used 3% nondenaturing cells. Lanes: **F,** free probe without protein; **B,** binding with extract protein; S, as in lane B plus 1,000-fold excess unlabelled homologous specific competitor; **NS,** as in lane B plus 1,000-fold excess Oct-1 binding site as a nonspecific competitor. Arrowheads indicate the free probe, and the arrows indicate bound complexes. **A:** Autoradiogram of EMSA for the indicated probes, which are described in Table I. **B:** EMSA was as in A. The thick upper arrow indicates a predominant low-mobility complex present only for the nt 7474 GREc.

cleotides for the nt 7385 and nt 7474 GREcs and HeLa cell extracts. The EMSAs showed two nt 7385 GREc–protein complexes (Fig. 3A, lane B) and three complexes with the nt 7474 GREc (Fig. 3B, lane B). Homologous specific (S) competitor completely abolished all binding, whereas an Oct-1 nonspecific (NS) oligonucleotide competitor had little effect. Moderately more binding was observed with the nt 7385 GREc, in comparison with the nt 7385 GRE oligonucleotide. The nt 7474 GREc and GRE showed more distinct binding patterns. The total protein binding to nt 7474 GREc in HeLa cell extracts was highly enhanced above that of the nt 7474 GRE. Furthermore, a predominant low-mobility complex uniquely appeared with the nt 7474 GREc oligonucleotide, whereas only two or three additional, faster-migrating EMSA complexes were observed with the other three probes (Fig. 3).

To characterize the proteins that were bound in the EMSAs, the GREc–protein complexes formed using HeLa extracts were also analyzed with Southwestern blot assays. Predominantly, a protein of the 96 kDa size of the GR subunits [Hollenberg et al., 1985] interacted with both probes (Fig. 4). These results were similar to the observations for wild-type nt 7385 and nt 7474 GREs [Mittal et al., 1993b]. Furthermore, the results suggested that the EMSA patterns were due to a higher level and

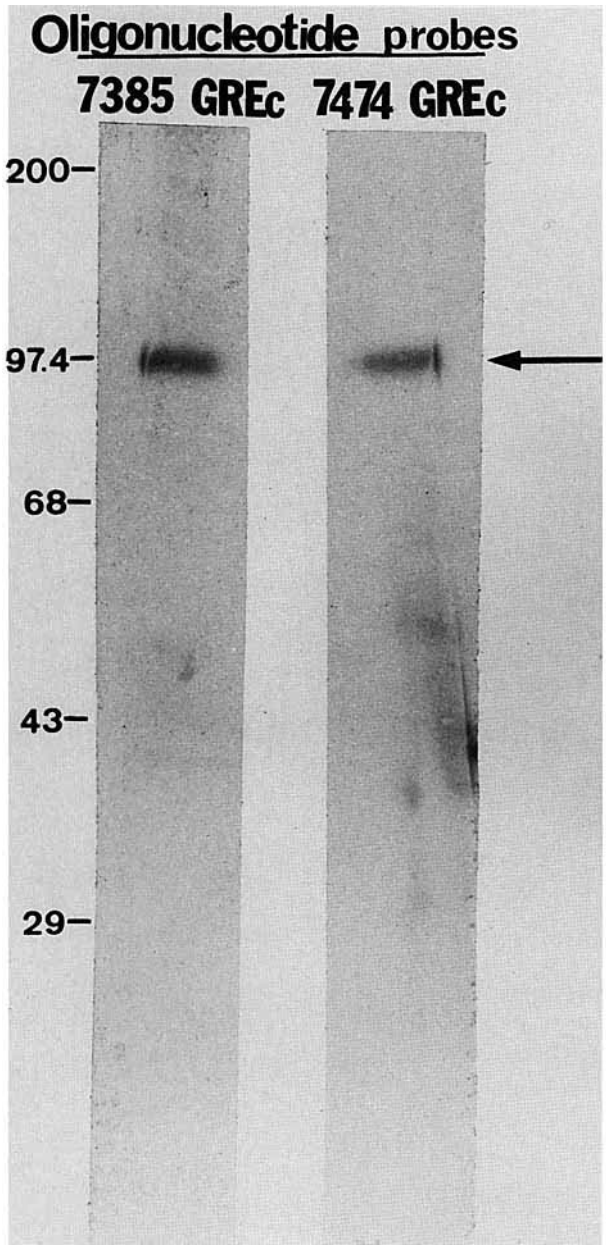

Fig. 4. Size of protein interacting with HPV16 GREcs. Southwestern blot assays of 100 μg HeLa whole cell extracts were used and were probed with 10^6 cpm/ml of the indicated end-labelled GRE oligonucleotides. The arrow indicates the putative GR 96 kDa. Protein markers are indicated in kDa on the left.

a unique multimeric form of the GR for the nt 7474 GREc, relative to the results with the three other oligonucleotides (Figs. 3, 4).

The Southwestern blot 96-kDa protein might form a heteromer or other protein–protein complexes, which could produce the EMSA patterns. To test such possibilities, UV crosslinking assays were used. The nt 7385 wild-type GRE and the GREc sequences were crosslinked to a protein of about 96 kDa (Fig. 5A). A few additional smaller bands were observed below 96 kDa and may thus represent proteolytic GR products, as reported previously [Singh and Moudgil, 1985; Mittal et al., 1993b]. The 96-kDa protein band was moderately more abundant with the nt 7385 GREc than with the corresponding GRE (Fig. 5A). A qualitatively similar result was observed for the nt 7474 GRE (Fig. 5B). However, a strikingly higher level of binding was observed to the nt 7474 GREc than to the corresponding GRE oligonucleotide. The results for both GREcs and both GREs were again substantiated with the specific and nonspecific competitors (Fig. 5). This differential binding was comparble quantitatively to that of EMSAs (Figs. 3, 5). Overall, the UV crosslinking assays show that a protein of the 96-kDa size of the GR binds to the GRE and GREc. Also, the affinity of GRE–protein interaction appears to be enhanced by the GREc mutations, especially for the nt 7474 GREc that also showed a highly enhanced level of complexes and distinct lower-mobilty complexes in EMSAs (Table III).

DISCUSSION

In addition to the association of HPVs with human cervical cancer, steroid hormones have been proposed as a cofactor [reviewed in zur Hausen, 1991; Pater et al., 1994; Khare et al., 1995]. Previous studies indicated that glucocorticoid hormone and progesterone mediate their effect through the GREs in the enhancer of HPV16 [Gloss et al., 1987; Chan et al., 1989; Mittal et al., 1993a,b]. In the present study, HeLa cells and HeLa cell extracts were used to examine the direct role of hormones. The possible complexities of regulatory controls and the DNA-binding patterns involved may be underestimated in the HeLa cell system, since PR acts through the same GREs as does the hormone-activated GR, and HeLa cells contain low levels of PR [Cato et al., 1986; Gronemyer, 1993]. HeLa cells support the expression of the epitheliotropic HPV16 enhancer [Gloss et al., 1987]. Further, this enhancer contains overlapping sites for GR, NF1, Oct-1, and AP-1, which additively or synergistically activate expression [Chong et al., 1991; Mittal et al., 1993a,b, 1994]. Previous studies showed that expression constructs of HPV16 GRE oligonucleotides gave a hormone response [Gloss et al., 1989; Mittal et al., 1993b]. A variety of different factors can be envisioned to be involved. To address such issues, GREcs were recently shown to be useful to analyze glucocorticoid-mediated expression from the regulatory regons of other genes [Rozansky et al., 1994; Jenab and Inturrisi, 1995]. Thus, we studied the role of glucocorticoid steroid hormones in HPV expression in HeLa cells with constructs containing HPV16 GREcs.

Generally, enhancers have complex structures [Mitchell and Tjian, 1989]. The unique and highly cell-specific HPV16 life cycle and process of oncogenesis apparently involve complex control by many factors [zur Hausen, 1977; Pater et al., 1994; Mittal et al., 1994]. The initial HPV16 GRE to be identified is at nt 7640 and is a composite GRE, since it contains an overlapping functional AP-1 binding site [Gloss et al., 1987; Chan et al., 1990a,b; Chong et al., 1990; Mittal et al., 1994]. Previously, DNA–

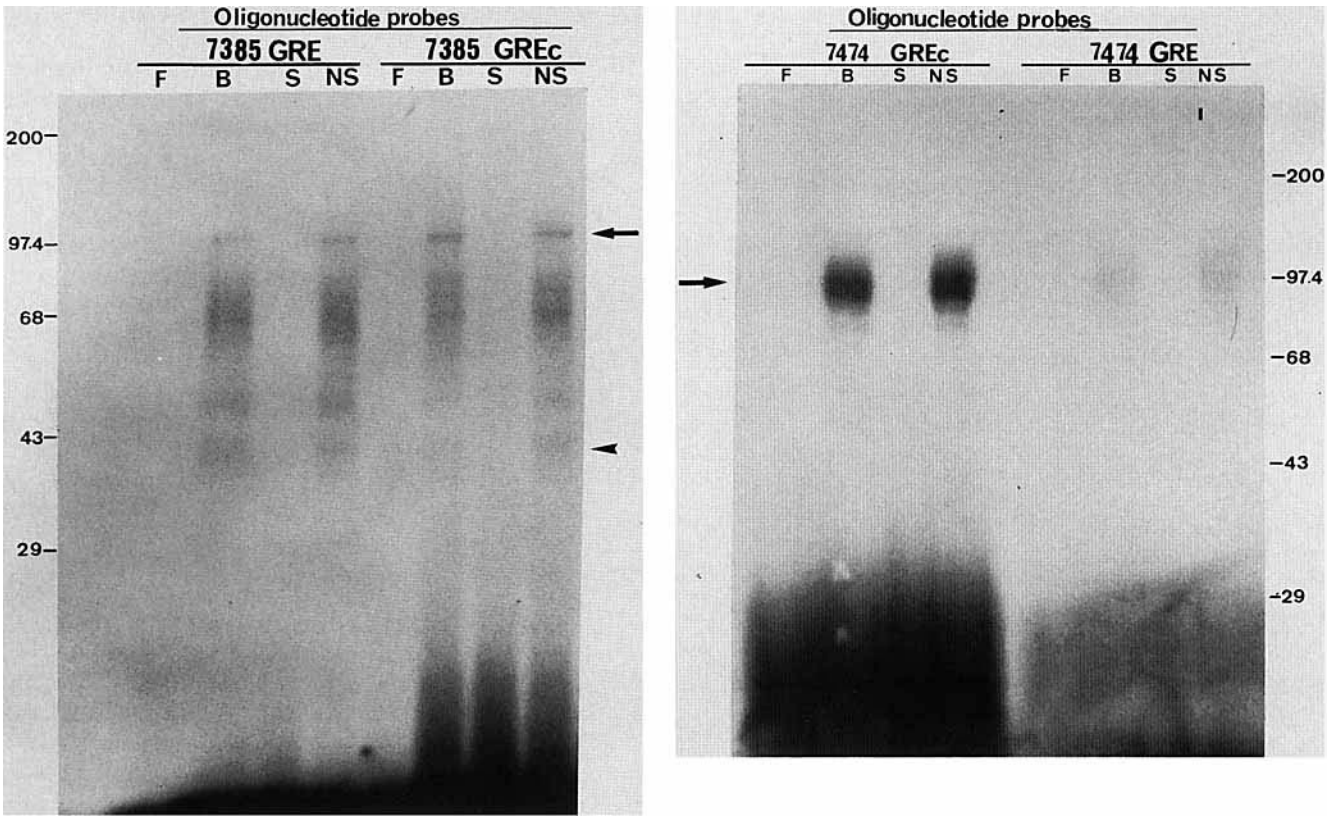

Fig. 5. Size and quantity of proteins interacting with HPV16 GREs and GREcs. UV crosslinking assays for 30 μg HeLa protein extracts were incubated with 10^5 cpm uniformly labelled oligonucleotide probes. The arrows indicate the putative GR. Other labels are as in Figure 4. **A:** Assays, as indicated, with the arrowhead indicating the putative GR degradation product. **B:** Assays with the markers indicated in kDa on the right.

TABLE III. Results of DNA–Protein Interaction Assays for GRE Oligomers*

	DNA–protein interaction assay		
Oligomer	EMSA	UV	SWB
7385 GRE	Two high-mobility complexes	43 and 96 kDa and intermediate signals	96 kDa
7385 GREc	Three unique complexes	More intense 96-kDa signal than for 7385 GRE	96 kDa
7474 GRE	Three unique complexes	Predominantly 96-kDa signal	96 kDa
7474 GREc	Two low-mobility complexes	More intense 96-kDa signal than with 7474 GRE	96 kDa

*Abbreviations used: EMSA, electrophoretic mobility shift assays; UV, ultraviolet crosslinking assays; SWB, Southwestern blot assays. The two GRE SWB results were reported previously [Mittal et al., 1993b].

protein interaction studies using HeLa nuclear extracts and the HPV16 enhancer region indicated specific binding of GR and AP-1 to the 7640 nt composite GRE [Chan et al., 1990a,b]. Such composite GREs were shown to be involved in complex regulation [Diamond et al., 1990; Mittal et al., 1994]. The complex control of gene expression arises through the interaction between transcription factors of two distinct signalling pathways via the composite GRE. The c-*jun* and c-*fos* proto-oncogenes in one pathway acted through the AP-1 site that overlaps the nt 7640 GRE of HPV16 to highly enhance the hormone response for GR in the second pathway [Mittal et al., 1994]. The nt 7640 AP-1 site is mutated in our GREc construct. However, GREc gave a highly enhanced induction by hormone, more than compensating for the loss of the enhancement via the AP-1 proto-oncogene

site. Therefore, the differential high hormone response probably is even more significant for the nt 7640 GREc. Generally, the positive regulatory role of both proto-oncogenes in hormone-induced expression through the overlapping AP-1 motif in the nt 7640 composite GRE [Mittal et al., 1994] suggested the importance of the activated GR in association with other regulatory factors. Adding to the complexity, is the independent regulatory mechanism of the cellular proto-oncogenes mediated through the AP-1 site [Chong et al., 1990; Peto et al., 1995; Nürnberg et al., 1995].

The nt 7385 and 7474 GREs were shown to be functional for oncogenic transformation [Mittal et al., 1993b]. Thus, the corresopnding GREcs should also be useful for studying the important role of hormones and HPV16 in the oncogenic conversion of normal human cervical cells and tissues, and the resistance to hormones of the premalignant and malignant counterparts [for review, see Pater et al., 1994]. Consensus sequences of the nt 7385 GRE and of the nt 7474 GRE containing an overlapping NF1 half site [Table I; Gloss et al., 1987; Sibbet and Campo, 1990] would further increase the expression of E6–E7 oncogenes as the binding of GR in response to hormone increases. The two GREs are more distal from the P97 promoter than the nt 7640 site. However, the loss-of-function mutation of the nt 7640 GRE had little affect on the hormone response for expression of HPV16 mRNA and transformation of BRK cells [Mittal et al., 1993a,b]. Further, additional complexities may involve interactions among the transcription factors binding to the flanking but not overlapping sites of the GREs, such as the NF1 sites and AP-1 motif [Pater et al., 1994]. For GR bound to the nt 7385 and 7474 GREs, enhancement of the binding could be through direct cooperative binding with GR on the nt 7640 GRE. Alternatively, the two sites may act by binding and, thereby, locally concentrating the cellular GR which reversibly dissociates from the three GREs.

Mutating the nt 7474 GRE to produce GREc also mutated the overlapping NF1 half site (Table I). Therefore, the EMSA binding pattern for the nt 7474 GREc was intriguing. There were different patterns of protein bound to the nt 7474 GREc relative to the nt 7474 wild-type GRE, and relative to those of the nt 7385 GRE and GREc. This suggests the importance of the NF1 site overlapping the nt 7474 GRE for protein–DNA binding and transcriptional activation by GR. Further experiments would be useful to address the role of the NF1 half site, with which NF1 is known to interact weakly or undetectably [Nakshatri et al., 1990; Chong et al., 1991; O'Connor and Bernard, 1995; Figs. 4, 5]. Complex cross-talk between the regulatory pathways and mechanisms of the NF1 and GR transcription factors may occur, analogous to the cross-talk shown for the composite regulatory elements of the HPV16 nt 7640 GRE/AP-1 site [Mittal et al., 1994] and of the NF1 half-site/Oct-1 site [O'Connor and Bernard, 1995].

Generally, enhanced binding of GR to GREs enhances expression [Dahlman-Wright et al., 1995]. The nt 7385 GRE has greater homology to GREc than does the nt 7474 GRE [Mittal et al., 1993b]. The difference in homology would contribute to the greater difference in the in vivo activity and in vitro binding to the nt 7474 GRE and GREc in response to hormone, relative to the nt 7385 GRE and GREc. Consistently, the activity of the nt 7385 GRE was greater than that for the nt 7474 GRE. Furthermore, the highly enhanced expression of the triple GREc construct may reflect enhanced cooperative interaction among the GR dimers bound to the three GREcs. Cooperative interaction was also shown for the MMTV LTR regulatory region, a paradigm for hormone-mediated regulation that contains a cluster of necessary high-affinity GREs [Beato et al., 1995]. The MMTV LTR is not only regulated by hormone via multiple GREs, but also shows NF1-dependent and -independent activation of expression by the GR [Mows et al., 1994; Lee et al., 1995]. Further, the whole HPV16 enhancer/promoter contains binding sites for many other transcription factors. For an additional level of complexity, HPV16 may be regulated by GR, PR, and the various other factors by cooperative interactions and via distinct mechanisms, interacting with GR or PR bound to GREs during viral infection and oncogenesis [Scheinman et al., 1995; Pater et al., 1994; zur Hausen and de Villiers, 1994; Mitchell and Tjian, 1989].

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